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Biocontrol action mechanisms of *Cryptococcus laurentii* on *Colletotrichum gloeosporioides* of mango

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ABSTRACT

Mechanisms of action and effectiveness of the antifungal yeast *Cryptococcus laurentii* [(Kuff.) C.E. Skinner] strain L5D, were examined against the causal agent of anthracnose *Colletotrichum gloeosporioides* ((Penz.) Penz. & Sacc.) in mango (*Mangifera indica* L.). *C. laurentii* showed a high antagonistic potential *in vivo*, with significant inhibition of anthracnose (75.88%). Different mechanisms of action were examined in *C. laurentii* among them competition for nutrients, specifically for sucrose ($p < 0.05$). Scanning Electron Microscopy (SEM) showed that the yeast biofilm adheres to the fruit and to *C. gloeosporioides* hyphae showing competition for space; *C. laurentii* was not washed off from treated *Colletotrichum* hyphae as observed with SEM. According to statistical analysis, only nagase and chitinase were significantly stimulated on wounded fruit. Activity of all three hydrolytic enzymes was detected *in vitro* but only nagase was induced by addition of autoclaved pathogen mycelium. Treated wounds with the yeast biocontrol stimulated glucanase activity and suppressed chitinase activity on fruit wounds with or without presence of autoclaved pathogen mycelium but did not affect nagase significantly ($p = 0.05$). Parasitic activity of yeast on pathogen was not detected.

1. Introduction

Postharvest diseases cause considerable losses during storage of fruits and vegetables (Janisiewicz and Korsten, 2002; Sharma et al., 2009). Anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Sacc. is a major postharvest disease of tropical fruits and vegetables (Jeger and Plumbley, 1988; Prusky, 1996), including mango (*Mangifera indica* L.) (Arias-Rivas and Carrizales, 2007; Bally, 2006; León, 2007; Vega-Piña, 2006). Its life cycle begins when conidia attach to the fruit surface, germinate and produce penetration structures. This pathogen causes quiescent infections and stays latent until fruit and environmental factors favor the development of the disease and the onset of symptoms. Disease symptoms may appear long after the initial stages of the infection (Arauz, 2000; Latunde-Dada, 2001; Mendgen and Hahn, 2002; Prusky, 1996; Prusky et al., 2013; Prusky and Lichter, 2007; Rodríguez-López et al., 2009).

The use of synthetic fungicides is the primary mean to control postharvest diseases of fruits. Public's growing concern for the negative health effects and environmental pollution associated with pesticide usage in orchards, the development of fungicide resistant strains of postharvest pathogens, and the lack of continued approval of some of the most effective fungicides are the impetus behind attempts to find alternative control methods (Droby et al., 2009; Jamalizadeh et al., 2011; Janisiewicz and Korsten, 2002; Sharma et al., 2009).

Biological control of postharvest decay of fruits using antagonistic yeasts has been explored as one of several promising alternatives to chemical fungicides (Liu et al., 2013; Rosa et al., 2010). In case of latent infection, biocontrol agents must be applied in preharvest on immature fruits, in addition to postharvest protection of wounds as maturation ensues (Ippolito and Nigro, 2000; Janisiewicz and Korsten, 2002). Different yeasts have been used to control *Colletotrichum* species. For example, *Cryptococcus magnus*

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(Lodder & Kreger) Baptist & Kurtzman (de-Capdeville et al., 2007) in papaya (*Carica papaya*); *Meyerozyma caribbica* (Vaughan-Mart. et al.) Kurtzman & Suzuki reduced incidence and severity of post-harvest anthracnose in mango (Bautista-Rosales et al., 2013); and *Candida membranifaciens* (Lodder & Kreger) Wick. & Burton significantly reduced severity of anthracnose on mango (Kefialew and Ayalew, 2008). Although, *Cryptococcus laurentii* has been reported as a biocontrol agent against fruit pathogens such as *Monilinia fructicola* (Winter) Honey in sweet cherry fruit (*Prunus avium* L.) (Wang and Tian, 2008); *Botrytis cinerea* (Pers.) in apple fruit (*Malus domestica*) (Yu et al., 2008); *Penicillium expansum* Link in jujube fruit (*Zizyphus jujuba* Mill.) (Cao et al., 2012); *P. expansum* and *B. cinerea* in pear fruit (*Pyrus pyrifolia* (Burm.) Nakai) (Yu et al., 2012); *Penicillium digitatum* (Pers.) Sacc. in mandarins (*Citrus reticulata* Blanco) (Guo et al., 2014), it has not been reported as a biocontrol agent of *C. gloeosporioides* in mango. The aim of this study was to evaluate the effect of the yeast, *C. laurentii* on anthracnose severity and to determine its mechanisms of action in mango fruits.

2. Material and methods

2.1. Microorganisms

The pathogen *C. gloeosporioides* was isolated from mango fruit (cv. Ataulfo). The fruits were obtained from commercial organic orchards of the municipality of San Blas, Nayarit, Mexico, a region recognized for its mango production, since for 2012 produced 17% of the mango fruit in Mexico with 249,802.66 tons, overcome only for Guerrero State (SAGARPA, 2014). This town is located at 21° 28′ N, 105° 10′ W and has an annual average rainfall of 1316.3 mm. *C. gloeosporioides* was isolated from mango fruits according to Chand-Goyal and Spotts (1996) and cultivated in potato dextrose agar (PDA). Plates were incubated at 28 °C for seven days and subsequently maintained at 4 °C for storage. A fresh culture was prepared on PDA prior to use in bioassays. For the bioassays, a conidial suspension (1×10^5 conidia/mL) was prepared on sterile distilled water using a hemocytometer.

The antagonistic yeast, *C. laurentii* (strain L5D), was isolated from the surface of 'Ataulfo' mango fruits (Bautista-Rosales et al., 2011) and grown in potato dextrose broth (PDB) on a rotatory shaker at 28 °C and 110 rpm for 72 h. Yeast cells were harvested by centrifugation at 3000 rpm for 10 min and washed twice in sterile distilled water to remove growth medium. Yeast cell pellets were resuspended in sterile distilled water, counted using a hemocytometer, and then adjusted to the desired concentration.

PCR was used to confirm the identification of both microorganisms (pathogen and antagonist). Fungal pathogen and yeast DNA was extracted by the technique described by Sambrook and Russell (2001). For *C. laurentii*, the divergent domain D1/D2 at the 5′ end of the large-subunit rRNA gene (Guadet et al., 1989) was amplified with primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) (Kurtzman and Robnett, 2003). For *C. gloeosporioides*, the ITS1-5.8s-ITS2 region was amplified with primers ITS1 (5′-TCCGTAGGTGAACCCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990).

PCR amplifications were performed in a thermal cycler (Applied Biosystem® GeneAmp® PCR System 9700, California, USA) with the following conditions: denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 30 s and a primer extension at 72 °C for 2 min; with a final extension at 72 °C for 10 min. Amplification products were separated by gel electrophoresis in 1% agarose (SIGMA-ALDRICH®, Germany) and stained with ethidium bromide (0.2 μg/mL), DNA amplicons were visualized on a transilluminator (BioDoc-IT system image, UVP®, USA) (Ochoa et al., 2007) and sequenced (Genewize Inc.), DNA sequences were aligned using the Basic Local Alignment Search Tool (NCBI BLAST) of the NCBI online database (http://blast.ncbi.nlm.nhi.gov/Blast.cgi) (Altschul et al., 1990).

2.2. Mango fruit

Mangoes (cv Ataulfo) were harvested at physiological maturity and with no apparent mechanical or microbiological damage. Fruits were harvested from an organic 20 year-old-trees orchard. Fruits were superficially disinfected with 1.5% sodium hypochlorite for two minutes, washed with tap water, allowed to dry at room temperature (28 °C) and finally placed in plastic containers (Casarrubias-Carrillo et al., 2002).

2.3. Effect of C. laurentii on anthracnose disease severity

Five healthy mangoes were wounded twice each with a bodkin (1 mm) depth. An aliquot of 25 μL from three-days-old yeast culture (1×10^7 cells/mL) were inoculated within the wounds and incubated for one hour. After the incubation, 25 μL of a *C. gloeosporioides* conidial suspension (1×10^5 conidia/mL) from a seven-days-old culture were added to the wounds. Five mango fruits were used as negative controls and treated with 50 μL of sterile distilled water within the wounds. To verify the non-pathogenic nature of the biocontrol agent, five fruits were inoculated with 25 μL of 1×10^7 yeast cells/mL and 25 μL of sterile distilled water as another negative control. Besides, five fruits were used as positive controls and inoculated with 25 μL of *C. gloeosporioides* conidia suspension (1×10^5 conidia/mL) and 25 μL of sterile distilled water. Treatments used to determine the effect of *C. laurentii* on anthracnose severity were: 1) fruits treated with yeast (strain L5D) at concentration of 1×10^7 cells/mL and *C. gloeosporioides* at a concentration of 1×10^5 conidia/mL, 2) fruits treated with *C. gloeosporioides* at a concentration of 1×10^5 conidia/mL, 3) fruits treated with sterile distilled water and 4) fruits treated with yeast (strain L5D) at a concentration of 1×10^7 cells/mL.

After treatments application, fruits were incubated in plastic chambers (30 × 40 × 15 cm) for six days at high relative humidity (>90%) and 28 °C (Taylor, Thermohygrometer 1455®, USA). One plastic chamber, containing five mangoes, was used per treatment. High relative humidity was kept by placing a 250 mL flask containing distilled water inside each chamber. At the end of the experiment, lesion diameter was measured per fruit to calculate the percentage of the disease inhibition according to the method of Benbow and Sugar (1999). A univariate design was used in which yeast inoculation was the factor and the diameter lesion was the dependent variable. The experiment was replicated twice (two wounds per fruit and five fruits per replicate) and repeated three times.

2.4. Nutrient competition

Competition for nutrients was determined by technique described by Spadaro et al. (2002) and modified by Magallón-Andalón et al. (2012). Briefly, five healthy mango fruits were washed, disinfected and drilled (0.7 cm in diameter and 1 cm deep) to evaluate the addition of different carbon sources and nitrogen sources in disease development. Lesion diameter (mm) in fruits was measured in presence of biological control agent with or without the addition of external nutrients such as 2% of sucrose, glucose or fructose as carbon sources or 0.3% potassium nitrate as the nitrogen source. Wounds were inoculated with 25 μL of the yeast suspension (1×10^7 cells/mL), 25 μL of a pathogen conidial suspension (1×10^5 conidia/mL) and 50 μL of a carbon or nitrogen

sources evaluated. In control treatments nutrients were replaced with 50 mM phosphate solution (pH 6.7). Fruits were incubated at 28 °C and >90% relative humidity for six days. At the end of the experiment, lesion diameters were measured. The experiment was repeated three times.

2.5. Adhesion of the yeast to the pathogen

Microcultures consisted of one cm^2 of *C. gloeosporioides* grown on PDA at 28 °C until mycelium was observed with the naked eye (72 h), according to Castoria et al. (2001) and modified by Magallón-Andalón et al. (2012). Thirty μL of *C. laurentii* or *Saccharomyces cerevisiae* cells adjusted to a concentration of 1×10^7 cells/mL were added to the mycelium. Mixed microcultures were washed with sterile distilled water after 12, 24 and 48 h so that only the yeast cells that adhered to the mycelium remained. Microcultures were observed using optical microscopy (40× and 100×) to examine a possible mechanism of parasitism. *S. cerevisiae* was used as a negative control.

Samples were prepared *in vitro* and *in vivo* to be examine by Scanning Electron Microscopy (SEM) (Hitachi S-3000N, San José, CA, USA). *In vitro* samples, consisted of *C. gloeosporioides* grown 72 h at 28 °C in PDB before an aliquot of 100 μL of *C. laurentii* (1×10^7 cells/mL) was added. The co-culture was incubated for another 72 h at the same conditions. Mycelium was fixed as outlined below. *In vivo* samples were prepared as described in section 2.3 (Reduction in the severity of anthracnose). Five fruits were incubated at 28 °C for 72 h. After that, a piece of pericarp tissue inoculated with yeast and *C. gloeosporioides* was excised from treated fruits with a scalpel (1 cm^3), cut in half and fixed.

SEM samples were fixed in 3% glutaraldehyde in cacodylate buffer 0.1 M (pH 7.4) for 72 h at 4 °C to preserve their structural and morphological characteristics, then the samples were placed in a solution of OsO_4 in 2% cacodylate buffer and processed as follows. Samples were rinsed three times in cacodylate buffer and dehydrated in a graded ethanol series (30, 50, 70, 80, 95 and 100%), critical point dried with CO_2 and coated with gold-plated for cell interaction assays. The specimens were examined by SEM (Hitachi S-3000N, San Jose, CA, USA) (Brown and Brotzman, 1979).

2.6. Hydrolytic enzymes secretion

Secretion of hydrolytic enzymes in the yeast was analyzed according to the methods of Castoria et al. (2001) and Magallón-Andalón et al. (2012). First, autoclaved *C. gloeosporioides* mycelium was produced to stimulate in the yeast the production of hydrolytic enzymes. *C. gloeosporioides* was grown in 50 mL of PDB and incubated at 25 °C with agitation (110 rpm) or until mycelial growth was observed. The culture was then autoclaved at 121 °C for 20 min and then centrifuged at 1400 × *g* for 10 min. The precipitate was washed twice with 50 mM phosphate solution (pH 6.7). Finally, it was resuspended in 50 mL of phosphate solution to stimulate the production of hydrolytic enzymes in yeast.

Enzymatic activity from *C. laurentii* was analyzed *in vitro* after growth for 72 h at 28 °C in yeast glucose broth in presence or absence of autoclaved *C. gloeosporioides* mycelium (10% V/V). Yeast cultures were centrifuged at 1400 × *g* for 10 min and filtered through a 0.20 μm nitrocellulose membrane. Supernatants were recovered for enzymatic analysis of β-1, 3-glucanase, N-acetyl-β-D-glucosaminidase (Nagase) and chitinase (Castoria et al., 2001; Magallón-Andalón et al., 2012; Spadaro et al., 2002). In order to test the induction of these enzymes *in vivo*, two wounds were made in five mango fruits (3 cm by 3 cm above and below the center of the fruit) with a bodkin (1 mm depth). One hundred μL of yeast suspensions (1×10^7 cells/mL) were inoculated into wounds. Additionally, a second treatment consisted of inoculating 100 μg of sterile *C. gloeosporioides* mycelium into the fruits. Fruits were incubated at 28 °C at >90% relative humidity for 72 h. Wounds were washed with 250 μL of 50 mM sodium acetate buffer (pH 5.0) for β-1, 3-glucanase enzyme, 50 mM phosphate buffer (pH 6.7) for N-acetyl-β-D-glucosaminidase (Nagase) enzyme and tris–HCl buffer (pH 7.5) for chitinase. Five replicates for each treatment were performed. After wound were washed, rinse solutions were collected and centrifuged at 1400 × *g* for 10 min and filtered through a nitrocellulose membrane (0.45 μm). The supernatant or enzymatic extract was recovered, and enzymatic activity was determined as described below. Control treatments consisted of mangoes which wounds were washed with 100 μL of sterile distilled water in the presence or absence of autoclaved *C. gloeosporioides* mycelium.

2.7. Determination of the enzyme activity

β-1, 3-glucanase activity was measured using the technique described by Castoria et al. (2001). Enzyme activity was quantified by measuring the nmol of reduced sugars released per mg of protein per min (mU/mg of protein). The N-acetyl-β-D-glucosaminidase (*Nagase*) activity was determined using the technique described by Tronsmo and Harman (1993) and was measured based on the nmol of p-nitrophenol released per mg of protein per min (mU/mg of protein). Chitinase activity was determined using the technique described by Wu et al. (2001) and was estimated as the nmol of p-nitrophenol released per mg of protein per min (mU/mg of protein). Finally, protein quantification was performed using a protein assay kit (Biorad, California, USA) based on the Bradford method (Bradford, 1976). A bifactorial statistical design was used to analyze our data, in which the first factor (variable) was the treatment (*in vitro*, fruits inoculated with yeasts and fruits non inoculated with yeast) and the second variable was with or without *C. gloeosporioides* mycelium.

2.8. Evaluation of the antibiosis mechanism

The antagonistic yeast strain was grown in YGB at 28 °C for 72 h until it reached a concentration of 1×10^7 cells/ml. Then, yeast culture was autoclaved for 15 min at 121 °C, centrifuged at 5600 × *g* for 10 min and filtered through a nitrocellulose membrane of 0.2 μm, to determine non-peptide antibiotics. To observe the effect of the enzymes on *C. gloeosporioides* development, one treatment was performed filtering the culture medium through a nitrocellulose membrane. An additional treatment was conducted where yeast cells were inoculated in order to determine whether they were necessary for the biological control capabilities. Twenty five μl of filtrates or yeast cells suspension were placed in a filter paper disk (Whatman #1) and deposited on a PDA plate that had been previously inoculated with 500 μL of *C. gloeosporioides* solution (10^5 conidia/ml). The plate was incubated at 25 °C for 72 h. The length of the inhibition halo around the disk was recorded. Simultaneously, a control plate was incubated in which a solution of 50 mM phosphate (pH 6.7) was applied instead of the sterile yeast culture. The treatments were performed in triplicate in accordance with Magallón-Andalón et al. (2012).

2.9. Statistical analysis

Data was examined using an analysis of variance (ANOVA). Data was analyzed using the SAS statistical software version 9.2 for Windows, and the least significant differences (LSD) test was used to separate differences among the means. Statistical significance was considered as $p < 0.05$.

3. Results and discussion

C. gloeosporioides, a mango fungal pathogen, isolate MA2 (GenBank Accesion number: JQ366003), and *C. laurentii*, yeast strain L5D, also known as *Cryptococcus flavescens*, *Rhodotorula flavescens*, *Rhodotorula aurea*, *Rhodotorula peneaus*, *Torula flavescens* or *Torulopsis flavescens* (ISF, 2014; ITIS, 2014; MycoBank, 2014), (GenBank Accesion number: JQ398672) were used through the experiments.

C. laurentii reduced significantly lesion development caused by *C. gloeosporioides* in a 75.88% compared to controls ($p < 0.05$). A reduction on the average of lesion diameter in the control fruits from 2.59 cm to 0.65 cm in fruits treated with *C. laurentii* (Fig. 1), demonstrated its antagonistic potential against *C. gloeosporioides*.

In vivo conditions, *C. laurentii* manifested different levels of competition for the substrates; however, only in the treatment with sucrose was observed an increase in the diameter of the lesion (Fig. 2). When sucrose was added in excess to fruits inoculated with yeast cells and the pathogen, an increase in the development of the disease by 68% was shown in relation with the treatment antagonist + pathogen ($p < 0.05$). This behavior occurs because both microorganisms were subjected to an environment with sufficient amounts of this substrate for their free development. Competition for this nutrient can be associated with the high inhibition exerted by the yeast on the pathogen, inasmuch as *C. laurentii* expressed a high degree of pathogen inhibition also competed for the primary carbohydrates present in the 'Ataulfo' mangoes in physiological maturity, which one of the most important is sucrose (0.2–0.5 g/100 g of sample) (García-Delgado et al., 2010; Montalvo et al., 2009). This finding indicates a high affinity and rapid assimilation of this carbohydrate by *C. laurentii*. Filonow (1998), Sharma et al. (2009) and Spadaro et al. (2010) mentioned that yeasts generally have the ability to successfully assimilate a wide variety of mono- and di-saccharides, such as sucrose, making these nutrients unavailable to *C. gloeosporioides* and allowing it to rapidly proliferate. Therefore, under conditions of starvation of sucrose, *C. laurentii* is able to compete with *C. gloeosporioides* for sucrose and to inhibit it in an efficient manner.

Adhesion of the yeast to *C. gloeosporioides* hyphae was observed by optical microscopy in mixed cultures after 48 h (Fig. 3 a and b). This phenomenon was not observed with *S. cerevisiae*, the negative control yeast (Fig. 3 c and d) because cells were rinsed off with sterile water. SEM showed that *C. laurentii* was able to form biofilms *in vitro* and *in vivo* (Fig. 4) and did not degrade *C. gloeosporioides* mycelium (Fig. 4 d).

Optical and scanning electron microscopy documented yeast adhesion to *C. gloeosporioides* hyphae (Fig. 4), which gives the yeast

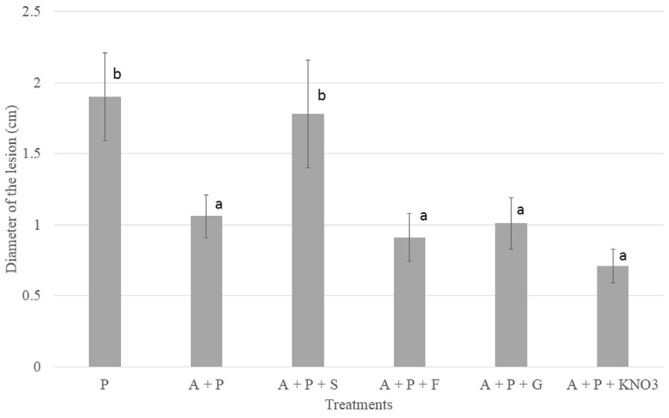

Fig. 2. Severity of *C. gloeosporioides* in wounded fruits of 'Ataulfo' mangoes treated with *C. laurentii* with addition or not of exogenous nutrients and inoculated with the fungal pathogen *C. gloeosporioides*. P: Pathogen, A: Antagonist, S: Sucrose, F: Fructose, G: Glucose, KNO_3: Potassium nitrate.

an important mechanism for the biocontrol of the fungus. Several previously reported studies mentioned that attachment is an important biocontrol mechanism of yeasts (Chanchaichaovivat et al., 2008; Hashem and Alamri, 2009; Long and Yuan, 2009; Saligkarias et al., 2002). Various studies have suggested that functional proteins from both antagonists and pathogen are involved in the adhesion process (Chan and Tian, 2005). Nevertheless, other studies indicated that this mechanism is related to nutrients competition in which yeasts interposed between the pathogen and the substrate, thus limiting pathogen growth (Bautista-Rosales et al., 2013; Sharma et al., 2009). During the adhesion process, some species of yeasts caused distortion of pathogen hyphae through the production of lytic enzymes such as glucanases, chitinases and proteases which degrade their cell wall and eventually cellular lysis is produced (Janisiewicz and Korsten, 2002; Sharma et al., 2009). In our studies, *C. laurentii* did not cause distortion or lysis of *C. gloeosporioides* hyphae (Fig. 4 d), suggesting that the adhesion process may be related to nutrients competition. Additionally, yeast was able to produce biofilms, which are communities of viable and nonviable microorganisms protected by extracellular polymeric substances (EPS) polyanionic fixed to a surface (Chmielewski and Frank, 2003). In this study, mature biofilms were detected *in vitro* and *in vivo* treatments (Fig. 4 a–c). Competition for space and nutrients, as well as metabolite production are potential mechanisms displayed against *C. gloeosporioides*, because yeast cells are primary colonizers excluding other potential colonizers

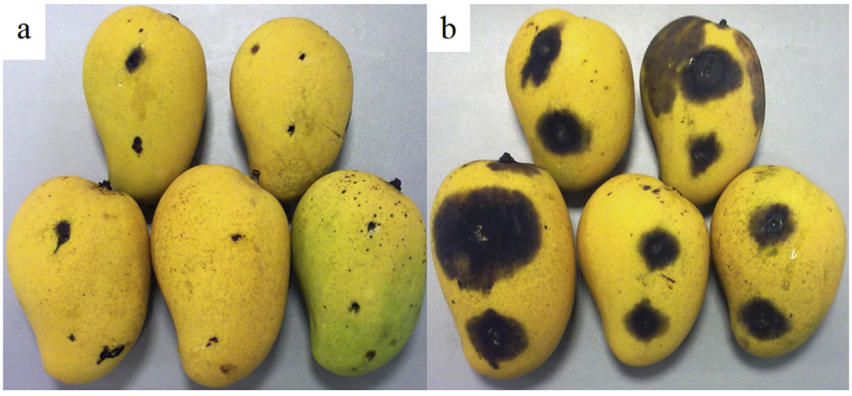

Fig. 1. Inhibition of *C. gloeosporioides* on mango fruits cv. Ataulfo. a) *C. laurentii*. b) Control treatment with distilled water.

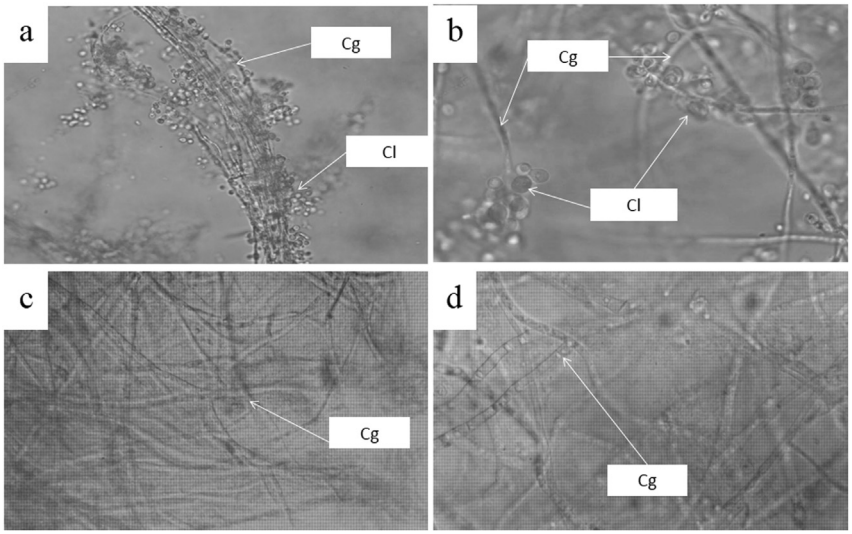

Fig. 3. Interaction between *C. gloeosporioides* and yeast after 48 h. Adhesion is observed by *Cryptococcus laurentii* on *Colletotrichum gloeosporioides* 40× (a) and 100× (b). No adhesion is observed by the negative control *S. cerevisiae* on *C. gloeosporioides* at 40× (c) and 100× (d).

(Lasa et al., 2005; San-José and Orgaz, 2010). The process starts with the adhesion of cells to the host through chemical signals and activation of producing EPS genes to form microcolonies (Fig. 4) (Navia et al., 2010; Visick and Fuqua, 2005). *C. laurentii*'s biofilm has been studied by several authors who mentioned that it is composed of protein, glucoronic acid and their potassium salt, as well as a heteropolysaccharide composed by monosaccharides such as arabinose, mannose, xylose, glucose, galactose and ramnose. This heteropolysaccharide has emulsifying and stabilizing properties which are advantageous when considering large-scale production and long term storage (Breierová et al., 2005; Jeanes et al., 1964; Pavlova et al., 2011).

No significant differences in β-1, 3-glucanase ($p > 0.05$) activity were found in yeast grown on PDB *in vitro*, with or without the addition of sterile mycelium (Fig. 5). Nevertheless, higher β-1, 3-glucanase activity ($p < 0.05$) were found *in vitro* compared *in vivo* experiments. Lower level of enzyme activity were found in wounded mango fruits non-treated with yeast with or without the addition of *C. gloeosporioides* mycelium vs. fruits inoculated with yeast with or without the addition of mycelium ($p < 0.05$).

N-acetyl-β-D-glucosaminidase (Nagase), levels were significantly higher in yeast grown on PDB (*in vitro*) in treatments that included the addition of *C. gloeosporioides* sterile mycelium ($p < 0.05$). Lower levels of nagase activity were detected in wounded fruits treated and non-treated with yeasts, a significant increase was detected with the addition of *C. gloeosporioides* sterile mycelium ($p < 0.05$) in experiments *in vivo*. A significant decreased in nagase activity was detected in fruits treated with the yeast compared to fruits non-treated with yeast either with addition or not of *C. gloeosporioides* sterile mycelium ($p < 0.05$) (Fig. 6).

No differences were found in chitinase activity of yeast grown *in vitro* in treatments with or without the addition of *C. gloeosporioides* sterile mycelium (Fig. 7), indicating that there was no activity induced in the yeast by the sterile mycelium. All fruits

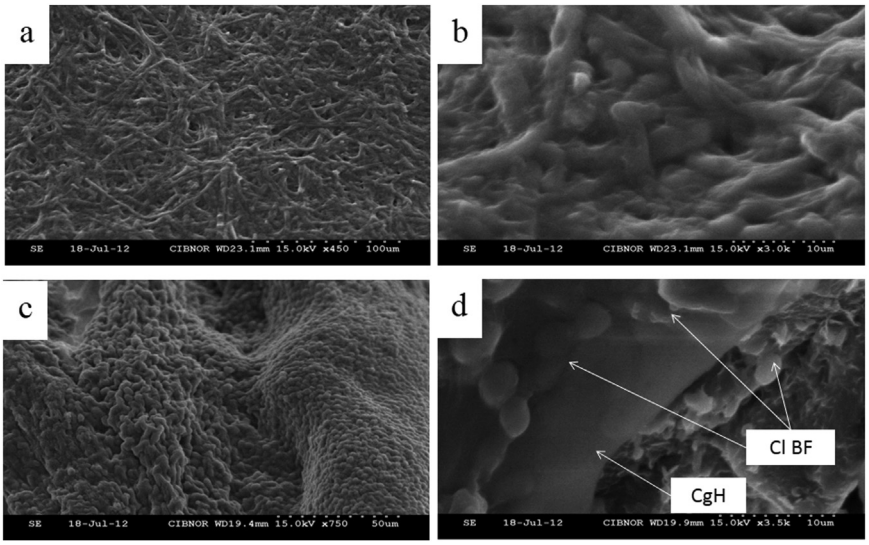

Fig. 4. Scanning electron micrographs (SEM) of the interaction between *C. laurentii* and *C. gloeosporioides* hyphae inoculated in PDB (*in vitro*) (a–b) and by direct pipetting on the Ataulfo mango wounds (*in vivo*) (c–d), where is observed *C. laurentii* biofilm (Cl BF) on *C. gloeosporioides* hyphae (CgH). Magnification of 450× (a), 3000× (b), 750× (c) and 3500× (d).

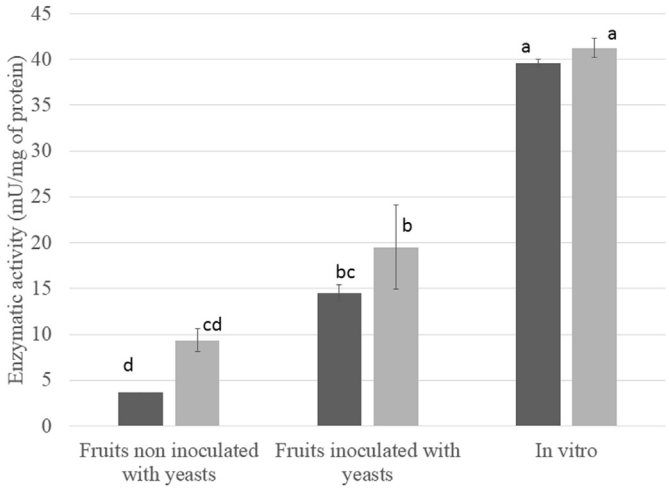

Fig. 5. Enzymatic activity of β-1, 3-glucanase *in vitro* by *C. laurentii* grown in PDB or *in vivo* by Ataulfo mango wounds and/or *C. laurentii* with addition ■ or not ■ of *C. gloeosporioides* sterile mycelium 3 days after inoculation.

treated with the yeast showed chitinase enzyme activity, but the addition of *C. gloeosporioides* sterile mycelium showed a significant increase in the chitinase activity in fruits non treated with the yeast and fruits treated with the yeast ($p < 0.05$), showing an induction of chitinase production. Lower levels were found among fruits treated with the yeast comparing with fruits non treated. This fact was observed with or without addition of *C. gloeosporioides* mycelium ($p < 0.05$). This behavior could be due to the exopolysaccharides produced by yeast and biofilm formation, decreasing the stress caused by fungi and reducing the production of chitinase.

According to results aforementioned, *C. laurentii* is capable of producing hydrolytic enzymes (glucanase, nagase and chitinase) *in vitro* and *in vivo*, although mango fruit by itself can also produce these enzymes in response to injury or detection of *C. gloeosporioides*. Statistically significant increase were observed in chitinase and nagase activity in fruits non treated with the yeast and enzymatic activity of nagase in fruits treated with the yeast when *C. laurentii* or mango fruit come in contact with the sterile mycelium of the pathogen. This situation is consistent with the findings reported by Chan and Tian (2005), Ma (2011), Pontier et al. (1998), Theis and Stahl (2004) and Vinale et al. (2008), who assert that either antagonist–pathogen or plant–pathogen interactions are due to a signal recognition system between the antagonist cells, the fungal hyphae and fruit cells. In contrast, in the case of β-1, 3-glucanase in control fruits and chitinase and glucanase in *in vivo* treatments, where *C. laurentii* was added, no statistically significant difference was observed in the enzymatic activity with or without the addition of sterile mycelium of the pathogen, although there was a slight increase in the activity of this enzyme.

Of all three different enzymes studied, no statistically significant differences in their activity were found in yeast grown *in vitro*, with or without the addition of sterile mycelium of the pathogen, suggesting that these enzymes are constitutive and part of their normal metabolism (Theis and Stahl, 2004).

In vivo tests of fruits treated with yeast, β-1, 3-glucanase activity increases in the presence of *C. laurentii* compared to control treatments (fruits no treated with the yeast), either in the presence or absence of sterile mycelium of pathogen. This phenomenon may be due to induction of fruit resistance response. *C. laurentii* induce resistance in tomatoes (*Solanum lycopersicum*) and jujube fruits (*Ziziphus jujube*) by the upregulation of genes related to metabolism, signal transcription, defense (β-1, 3-glucanase, endo-β-1, 4-glucanase or PR−1a1) **and stress** (wound-induced protein and meloidogyne − induced giant cell protein) (Jiang et al., 2009; Tian et al., 2007).

On the other hand, during evaluation of the production of antifungal compounds, *C. laurentii* did not produce inhibition halos neither in the non-peptide antibiotics test (filtered and sterilized extracts) nor in the enzymatic extracts (filtered) (Fig. 8). However, when the inoculation was performed with yeast cells, there was not mycelial growth in the area where yeasts grew.

This observation may indicate that there was no production of antibiotics or the amount produced was not enough to produce a significant inhibition. A similar response occurred when the filtered extracts without sterilization containing the previously mentioned hydrolytic enzymes were used. This result suggests that, in the absence of antibiotics and/or enzymes in sufficient quantity, there is no inhibition of the development of *C. gloeosporioides*. However, when the yeast cells were inoculated in the filter paper discs and placed on PDA with *C. gloeosporioides*, there was a clear inhibition of the fungus (Fig. 8c), i.e., this yeast likely has several mechanisms for inhibiting the development of *C. gloeosporioides*, and it is the

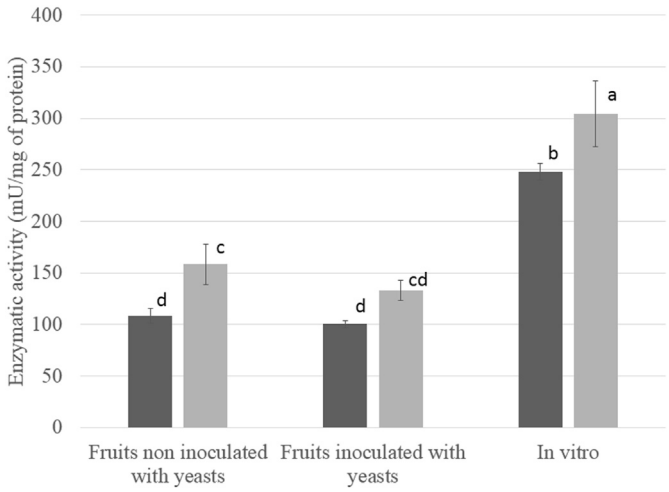

Fig. 6. Enzymatic activity of N-acetyl-β-D-glucosaminidase (nagase) *in vitro* by *C. laurentii* grown in PDB or *in vivo* by Ataulfo mango wounds and/or *C. laurentii* with addition ■ or not ■ of *C. gloeosporioides* sterile mycelium 3 days after inoculation.

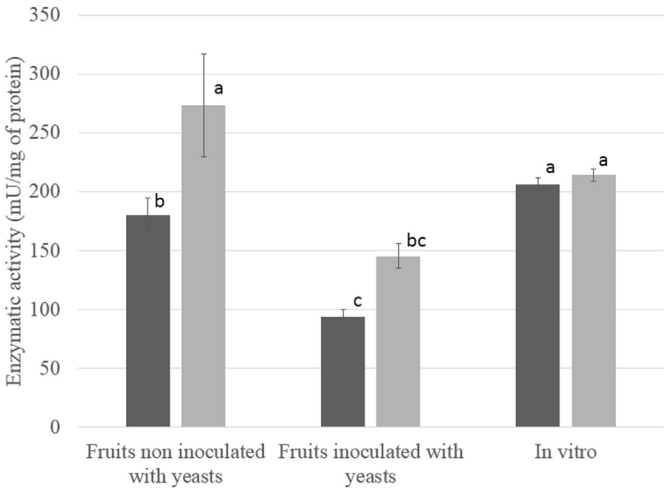

Fig. 7. Enzymatic activity of chitinase *in vitro* by *C. laurentii* grown in PDB or *in vivo* by Ataulfo mango wounds and/or *C. laurentii* with addition ■ or not ■ of *C. gloeosporioides* sterile mycelium after 3 days after inoculation.

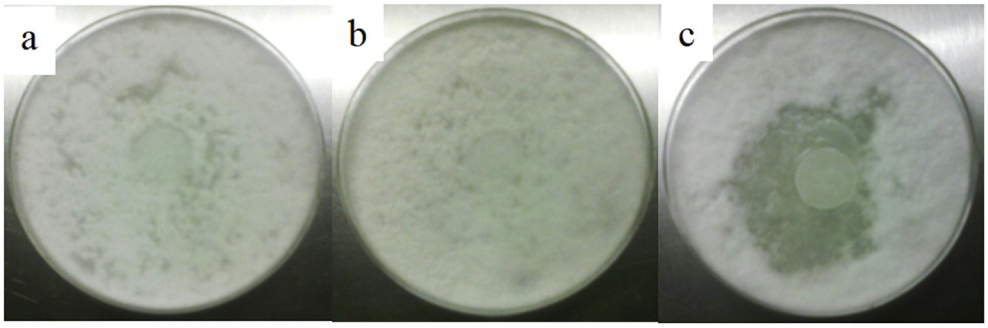

Fig. 8. *C. gloeosporioides* grown in PDA with addition of filtered and sterilized extract (a), filtered extract (b), and cells of *C. laurentii* (c).

combination and/or synergism of mechanisms that provides the antagonistic capacity for this process as previously stated by Ippolito et al. (2005) and Janisiewicz and Korsten (2002).

Results of this study clearly showed that *C. laurentii* can reduce anthracnose when yeast cells were applied directly to mango fruit, showing that *C. laurentii* has several action mechanisms that can be used to manage mango anthracnose caused by the fungus *C. gloeosporioides*, *C. laurentii* has the advantage over synthetic fungicides that having multiple ways to attack (biofilm production, production of hydrolytic enzymes, etc.) and/or compete (competition for nutrient and space) against a pathogen reduces the risk of induced resistance (Guédez et al., 2009; Janisiewicz and Korsten, 2002; Van-Lenteren, 2008). However, further research is required 1) To conduct genetical and chemical studies to knockout specific biochemical pathways, 2) to develop technology to increase effectiveness of the biocontrol yeast species studied, 3) to ensure the complete safety of *C. laurentii* strain L5D to animals and humans, 4) to evaluate *C. laurentii* strain L5D with others fruits, and 5) to optimize growth media for large-scale production that enables field applications according to the demands of mango cultivators and especially for those engaged in the organic production.

4. Conclusions

The yeast *C. laurentii* L5D is an efficient biocontrol agent of *C. gloeosporioides* the causal agent of anthracnose. This yeast exerts several mechanisms of action such as competition for space and nutrients, production of hydrolytic enzymes, induction of resistance and biofilm formation. To ensure the effectiveness of its antagonism, it is necessary to use a formulation in which yeast cells are applied directly to fruit.

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